Inducing Somatic Embryogenesis in Grape (*Vitis aestivalis* 'Norton/ Cynthiana') Tissue Callus Derived from Ovary Explants

By

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Abstract

The grape plant *Vitis aestivalis* "Norton/Cynthiana" is known for its hearty nature and low maintenance. However, this grape is also known for its poor propagation. Plant tissue culture is a method that has been used to propagate other recalcitrant species. Somatic embryogenesis in *Vitis aestivalis* has been attempted using callus generated from leaf explant tissue, but to date, has not been successful. The use of floral tissues has shown some success in other grape species, so the first goal of this research was to generate undifferentiated cell growth, or callus, from anther and ovary tissues of immature flower buds. Callus growth was successfully achieved using a Lloyd McCown basal nutrient tissue culture medium. Healthy callus tissue was quickly created from the ovary tissue, but callus was not immediately successful from the anther tissue. Anther explant tissues had to remain on the media for several weeks longer than expected before callus began to grow. The second goal of this project was to successfully generate somatic embryogenesis from callus. Both the anther and ovary callus were placed on embryogenic tissue culture media in an effort to promote embryogenesis. As *Vitis aestivalis* is not easily propagated, the embryogenic tissue media had to be carefully made and adjusted to find the exact mix of cytokinin and auxin concentrations that will lead to embryogenesis.

In this project, no embryogenic response from ovary tissue on either the semi-solid media or liquid suspension media was observed. Semi-solid media were shown to be good for regular maintenance of callus, but not embryogenesis. Liquid suspension culture also proved effective for callus maintenance, but not embryogenesis. The use of

Murashige & Skoog basal salts seemed to advance callus growth in the liquid suspension media.

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Introduction

Vitis aestivalis 'Norton/Cynthiana' is native to North America and is known in the wine industry as a high-quality grape. The plant is well known for its disease-resistant and hardy nature. One of the most noteworthy characteristics of the grape is the presence of a fungal endophyte inside the plant. The fungal endophyte's presence in the plant is believed to play a role in its ability to maintain protection against disease, insects, and drought (Uhls, *et al.*, 2018).

However, the 'Norton/Cynthiana' grape also is known for its poor propagation. Traditional methods of propagation have a success rate of about 10-20%, yet the wine grape's hardy characteristics and high-quality wine still make it a desirable grape variety in the wine industry. Propagation through seeds and cuttings have proven to be unreliable and undependable, thus the movement to other techniques of propagation (Wilson *et al.*, 2016). It is hoped that tissue culture technology can be used to improve the low propagation success rates for this grape variety. The technology of plant tissue culture was facilitated in this experiment with both semi-solid and liquid media.

Tissue culture media were developed to create a mass of undifferentiated cells called callus. Media with different levels of chemical components can produce undifferentiated growth from the grape tissue. Those undifferentiated cells can then be used in an effort to produce somatic embryogenesis (Carra *et al*., 2016). Though many species of grape have been used to produce callus that will result in successful embryogenesis, few strains have successfully been brought to embryogenesis (Dhekney *et al*., 2009). Due to the uniqueness and individuality of each grape variety, different

levels of hormones, vitamins, and salts have been tried in order to find the perfect mixture that allows for embryogenesis.

In contrasting studies of embryogenesis from grape tissue culture, different parts of the grape plant were used (Alavijeh *et al*., 2016). The purpose was to find which parts of the grape plant produce callus, and which do not. Subsequently, this study identified which parts of the plant are able to produce viable callus and, therefore, embryogenesis. The short flowering time, found to be typically around the last week of April and the first week of May, creates a small window for the collection of flower tissue (Kikkert *et al.*, 2004). A previous study on the 'Norton/Cynthiana' grape proved to be successful in the creation of undifferentiated cell growth by leaf explants via tissue culture (Wilson *et al*., 2016). This study was immediately followed by that of Hall (2018), who varied the age of the callus and the amounts of plant growth regulators and types of salts used in the media. As Hall's study suggested, the success of somatic embryogenesis could be dependent on the explant tissue used or the methodology of callus creation and maintenance used.

In the current research, the ovary found inside the pre-flowering grape buds was isolated in order to create a mass of undifferentiated cells, or callus. Following the successful creation of undifferentiated cells, the callus was applied to different types of media to attempt to generate plant embryos. Alongside the use of solid media types, liquid suspension was also utilized to achieve embryogenesis from the grape ovary callus. The main objective of this study was to find the media type that would successfully reach multicellular growth, leading to embryogenesis.

Materials and Methods

Media Composition

The callus-inducing selection medium contained Daconil® (chlorothalonil), which is an anti-fungal chemical used to prevent fungal growth. The medium's basal ingredients included 1.205 g of Lloyd McCown woody basal salts, 15 g of sucrose with 500uL of thiamine and 200 uL of casein in a total of 500 mL deionized water. Both hormones 2,4-D and kinetin were added at 1.356 μM and 0.93 μM, respectively. After being thoroughly stirred together, the medium's pH was set to pH 5.6-5.7 using 0.5 M KOH. Once the proper pH was reached, four and a half grams of agar were added to the flask and autoclaved at 121°C for 20 minutes. After autoclaving, the flask was left on a stirring plate for approximately 40 minutes until cooled. Eight-hundred-seventy-five microliters of Daconil® was added to the cooled solution and left to mix. The medium was then taken to a biosafety hood, where it was poured into 100x15 mm sterile Petri dishes. The dishes were left in stacks of two or three in the hood to solidify over the course of an hour. Once completely solidified, the dishes were wrapped with parafilm to remain sterile and placed in a refrigerator. The regular maintenance medium followed the same process and same ingredient list without the addition of Daconil® before plating.

A semi-solid embryogenic medium was created by adding 1.205 g of Lloyd McCown Woody basal salt, 15 g of sucrose, 500 uL of thiamine, and 200 uL of casein to 500 mL of deionized water. Finally, 5 g of activated charcoal was added. The desired pH was 5.6-5.7 and the initial pH of the medium was found to be around 6. Due to pH fluctuations caused by the addition of the activated charcoal, 500 uL of 1 M HCl was added to the medium and the medium was left overnight on a stirring plate. The next day,

the pH was around 4.5 or higher. At that point, potassium hydroxide (KOH) was pipetted into the medium while it remained on a stirring plate until the pH remained constant at 5.6-5.7. Once the proper pH was reached, 4.5 g of agar were added and autoclaved at 121°C for 20 minutes. After the medium was cooled, it was plated in the same manner as previously described.

Liquid Suspension Media

The first liquid suspension medium made was meant to mimic the regular maintenance medium. The same basal components, with the exception of agar, were added in the same amounts in a 500 mL solution. Due to the increase of surface area around the callus, the hormone amounts were reduced to 0.678 μM of 2,4-D and 0.465 μM of kinetin. The pH was maintained at 5.6-5.7 as with the other media, and it was autoclaved at 121°C for 20 minutes. The medium was stored in an Erlenmeyer flask in a refrigerator with the mouth covered with aluminum foil to maintain sterility.

A second liquid medium contained all the same basal ingredients used in the maintenance liquid suspension medium and created in the same manner following the same steps. However, after it was taken from the autoclave and cooled, 0.444 μM of 6-benzylaminopurine (BAP) was added to the solution. This liquid medium was the primary liquid suspension embryogenic medium.

A third liquid medium contained the same basal ingredients used in the maintenance liquid suspension medium and followed the same steps. After autoclaving and cooling, 0.222 μM of BAP was added to the solution. This liquid medium was the secondary liquid suspension embryogenic medium.

A fourth liquid medium was made using 1.205 g of Murashige & Skoog basal salt, MS salt, instead of 1.205 g Lloyd McCown basal salts. Other than the salts, the basal ingredients remained the same. After autoclaving and cooling, 0.444 μM of BAP was added to the solution. This liquid medium was the tertiary liquid suspension embryogenic medium.

Tissue Collection and Sterilization

Flower buds were collected from the Rutherford County Agricultural Exposition/MTSU Vineyard around the final week of April and first week of May, 2018. Once collected, they were surface sterilized in a biosafety hood by washing the whole flower bud in a solution of 70% ethanol for thirty seconds. Then, the buds were transferred to sterile deionized water, where they were rinsed for thirty seconds. Following the water rinse, the tissue was cleaned in a 10% bleach solution that contained 1% surfactant (Dawn® dish soap) for ten minutes while being continually agitated. After ten minutes, the sterile tissue was rinsed for five minutes with sterile deionized water three times.

After being sterilized, the still-closed buds were taken to a sterile biosafety hood. With a sterile blade and tweezers, they were dissected under a dissecting microscope inside the biosafety hood. The outside protective layer was removed by cutting at the base of the sepal and peeling the tissue away from the center structure, composed of the ovary and anthers. A sterile blade and tweezers were used to pull the attached anthers away from the ovary tissue. Also removed from the ovary of the plant were the stigma and style portions.

Plating

Immediately after dissection, the explants were placed on callus-inducing selection media in three by three rows. The plates were incubated at 25°C for no longer than a week as the medium contained Daconil®, a chemical which becomes overwhelming and toxic to the explants after too much exposure. The explants were then transferred to the regular maintenance medium where they were left to develop callus.

Liquid Suspension Set Up

After healthy, white callus was developed on the plates of maintenance media, three aliquots, one half centimeter in diameter, were taken from the maintenance medium and placed in 250 mL flasks. Maintenance liquid suspension media was pipetted into the flask until the meniscus reached 100 mL. The flask was then placed on a shaker at 100 RPM to maintain constant agitation. After approximately 5 days of agitation on the shaker, 20 uL of the slush was removed and added to 30 uL of the chosen liquid suspension embryogenic media types. They were maintained at 50 mL of 2 parts callus slush from the maintenance liquid suspension media to 3 parts liquid suspension embryogenic media.

Results

Fungus-free callus was successfully created using the callus-inducing selection medium. On that medium type, the fungal-free callus was maintained on the regular semi-solid maintenance medium (Figure 1). Healthy, white callus was generated regularly on the maintenance medium when transferred every two weeks.

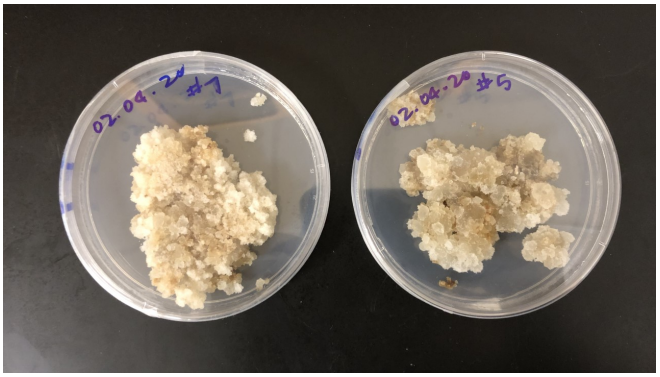

Figure 1: Healthy ovary callus generated on semi-solid maintenance media

The embryogenic semi-solid media type that used activated charcoal generated healthy, white callus and was used to revive the callus when it became too dry or browned (Figure 2). The medium did not lead to embryogenesis.

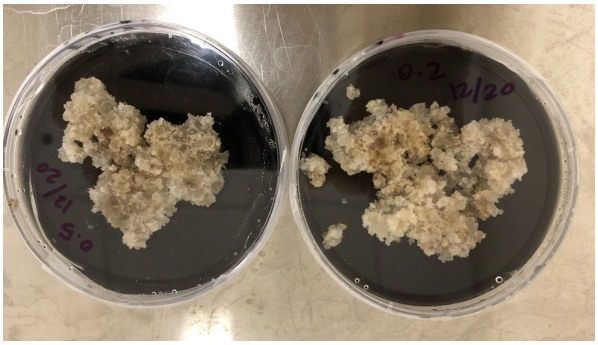

Figure 2: Healthy callus development on activated charcoal embryogenic media

The first liquid suspension medium used that mimicked the regular maintenance medium generated callus on its own while on the shaker (Figure 3,4). The callus remained healthy for approximately two weeks before displaying signs of stress and death. Signs of stress include browning and thickening of the suspension. Embryogenesis with this medium was not successful.

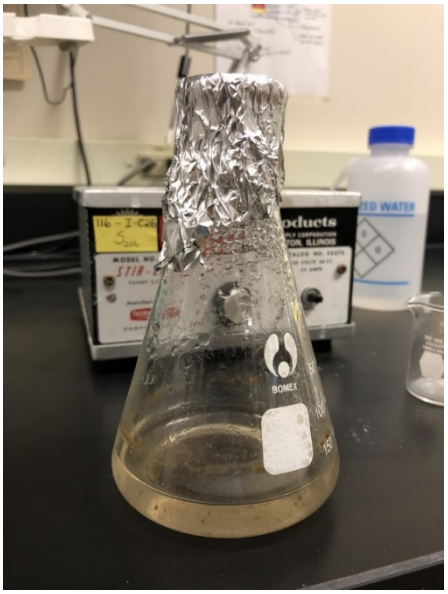

Figure 3: Liquid suspension maintenance media

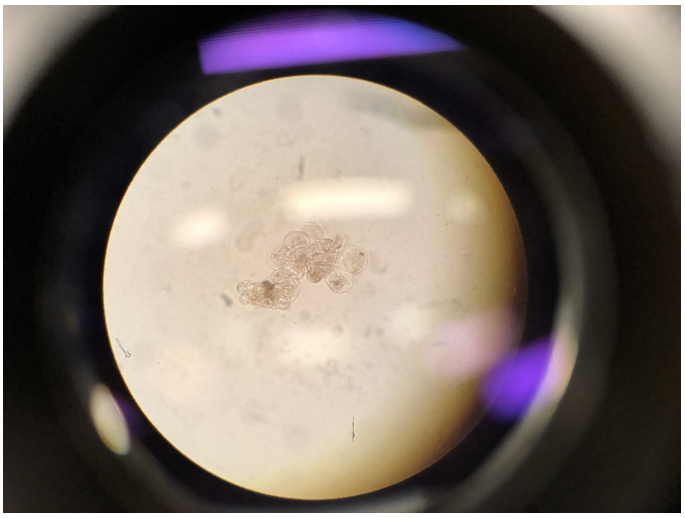

Figure 4: Healthy callus growth in liquid suspension maintenance media

The second liquid suspension medium used that contained 0.444 µM of BAP did not lead to the creation of embryos. The callus was only able to remain in this medium for approximately one week before rapidly browning with high cell death (Figure 5). However, this medium displayed bicellular growth, indicating that multicellular growth could be occurring in the liquid suspension media (Figure 6).

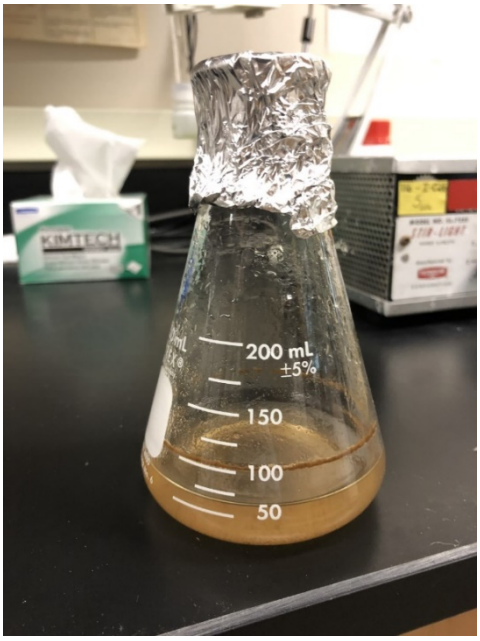

Figure 5: Liquid suspension with 0.444 µM of BAP

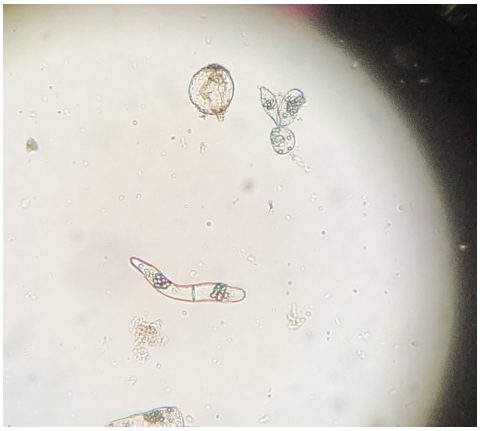

Figure 6: Bicellular growth found in the liquid suspension

The third liquid suspension medium that used 0.222 µM of BAP did not lead to embryogenesis, though the medium produced more rapid cell growth with slowed browning (Figure 7). The medium quickly developed balls of callus in the bottom of the flask and the medium remained a misty white color that indicated good cell growth (Figure 8).

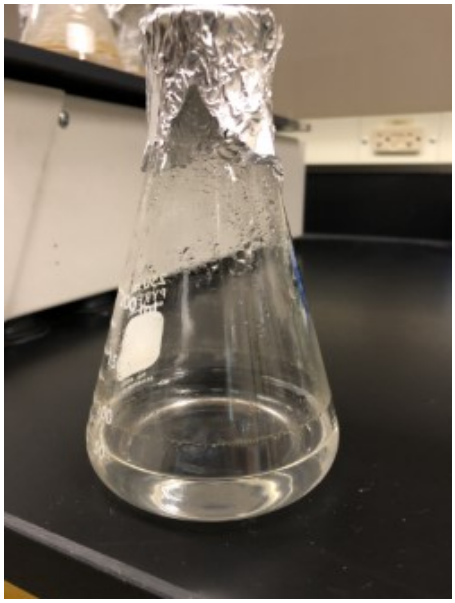

Figure 7: Liquid suspension media with 0.222 µM of BAP

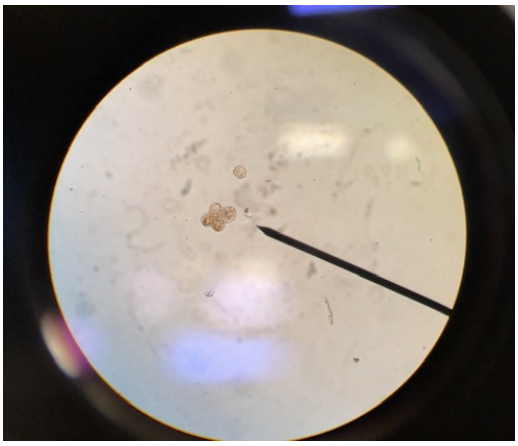

Figure 8: Callus growth in liquid suspension media with 0.222 µM of BAP

The fourth liquid suspension media type that used MS salts and 0.444 µM of BAP did not lead to embryogenesis, but showed better cell growth with slowed browning (Figure 9). This medium also maintained healthy balls of callus developing in the bottom of the flask and the medium remained a misty white color indicating healthy cell growth (Figure 10).

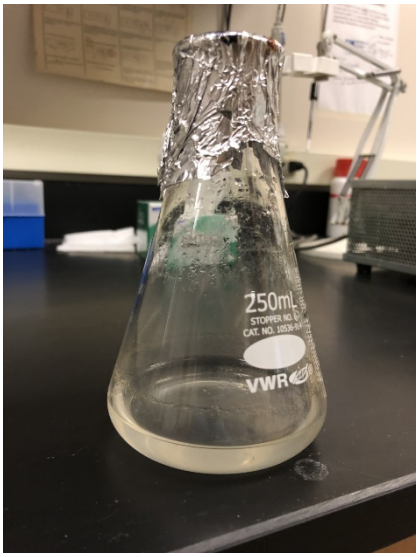

Figure 9: Liquid suspension with 0.444 µM of BAP and MS salt

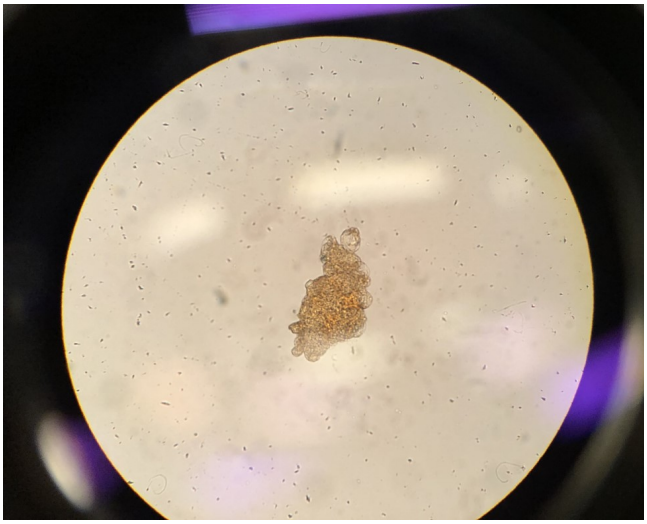

Figure 10: Callus growth in liquid suspension with 0.444 µM of BAP and MS salt

Discussion

This study sought to determine whether ovary explant used in undifferentiated cell growth could yield somatic embryogenesis in *Vitis aestivalis*. The ovary explant tissue was used in the same manner as the leaf tissue was used in the Hall (2018) study and followed the suggestions therein for using liquid suspension as the next step in attempted embryogenesis.

While both the semi-solid callus-inducing medium and maintenance medium proved to be successful in the creation of callus via the ovary explants, the semi-solid media used for this study were used for regular growth and maintenance only.

The liquid suspension treatments were designed after the semi-solid media type treatments used by Hall (2018). Growth hormones and different salts were changed in the liquid suspension medium in an attempt to find a combination that could lead to embryogenesis. The change to from Lloyd McCown woody basal salts to Murashige & Skoog basal salts showed a reduction in cell death within the time the flasks remained on the shaker. The presence of more than 50 uL of BAP suggests more harm than good in the liquid suspension culture, whereas, 25 uL seems to promote a sustainable environment for cell growth.

A difficulty faced within the study was the ratio of callus to liquid suspension media. Too much media with insufficient callus did not show much growth, while too much callus displayed rapid cell death and browning of the solution. The best ratio appeared to be around 200 mL:300 mL callus to media, but cell growth remained slow.

One of the biggest issues revealed in the study was the age of the callus. The ovary tissue was gathered in April of 2018, making it almost two years old. As the Hall (2018) study suggested, the younger the callus, the better the cell elongation. Younger callus may respond better to the liquid suspension treatment with the use of ovary explant tissue, making somatic embryogenesis possible with newer callus.

Further research should utilize younger callus since they respond better to liquid suspension media. The age of callus needs to be tracked, similar to the Hall (2018) experiment, as liquid suspension media is used to reach embryogenesis. In addition, liquid suspension media may be more responsive to multicellular growth when MS salts are used instead of Lloyd McCown salts.

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